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본시멘트를 이용한 생체활성 하이드록시아파타이트 미립구의 제조 및 평가

Bioactive Hydroxyapatite Microspheres Obtained from Bone Cement for Biomedical Application

2014년 2월

서울대학교 대학원

재료공학부

백재욱

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지도교수 김현이

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백 재 욱의 석사 학위논문을 인준함

2014년 2월

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Abstract

Bioactive Hydroxyapatite Microspheres Obtained from Bone Cement for Biomedical Application

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With increasing demand of bone substitute, bioactive ceramics have been widely used in dentistry and orthopedics. In particular, hydroxyapatite (HA) has been increasingly getting attention as a promising implant material for bone defect due to its great biocompatibility. Despite many superior properties of HA, dense HA implant is limited owing to low biodegradability and lack of enough space for new bone to ingrow. Microsphere-type materials allow injectability which is minimally invasive procedure and bone ingrowth through the interspacing built among spheres. To accelerate bone healing rate, drugs or bioactive proteins are usually incorporated to ceramics, but thermal treatment of ceramic processing limited it.

To overcome the problems, bone cement is introduced to make HA microspheres since room temperature processing is possible for bone cement. Researchers have already made microspheres from bone cement, but the

products have low efficiency in fabrication time and drug loading. Previous studies using α-tricalcium phosphate (α-TCP) needed one week of incubation in simulated body fluid (SBF) solution to achieve mechanical and chemical stability. This is problematic for two reasons. First, it takes too long for researchers. Second, if drugs were loaded in the microspheres, a large amount of drugs can be released away during one week of incubation.

In the first study, to get mechanical stability without SBF incubation, tetracalcium phosphate(TTCP) and citric acid were added to previous system as bone cement powder and hardening additive respectively. To get chemical stability within only a few hours, 10x SBF-like solution was used to coat the surface of microspheres with apatite. So, the final product is surface modified calcium phosphate microspheres with mechanical and chemical stability. TTCP played an important role in making difference of hardness. TTCP dissolved quick and filled the space among undissolved α-TCP particles since it is 10x more soluble than α-TCP. Furthermore, *in vitro* cell test using pre-osteoblast cells, the apatite coating layer formed in 10x SBF like solution showed enhanced biocompatibility compared to bare surface. It was meaningful to achieve better product within significantly recued time.

Even though in the first study fabrication time was noticeably saved, it could be not adequate in drug delivery applications because there are a few hours of immersion, which causes drug loss to the medium. So, it is hard to approximate the amount of drug loaded and inappropriate for biomedical

industry which requires accurate dose of drug. To avoid incubation in hydrophilic medium and achieve chemical stability at the same time, microspheres were kept in the oil, allowing them to be converted to HA with moisture originated from hardening liquid. After 3days, large amount of reactants were consumed and phase was transformed mainly to HA. For the advantage of visualization, green fluorescent protein (GFP) was used instead of drugs or growth factors. GFP was well distributed in the microspheres and its release behavior seemed to be sustained at least one month. To verify the potential use in the biomedical field, preliminary *in vivo* animal test was performed with a rat. BMP-2 was loaded in the microspheres and it showed better osteoconduction and osteoinduction compared to microspheres without BMP-2.

In conclusion, these two experiments were focused on fabricating stable calcium phosphates microspheres with high efficiency in time and in drug loading. *In vitro* cell test showed rapidly-formed apatite coating layer had effectively enhanced biocompatibility. Also, preliminary *in vivo* animal test verified that drug loaded calcium phosphate microspheres converted in oil have great potential to be used as a bone filler.

Keywords: Microspheres, Hydroxyapatite (HA), Calcium phosphate bone cement, Water/oil emulsion, Sustained release, Bone regeneration, in vivo.

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Chapter 1.

Introduction

(Theoretical review)

1.1. HA microspheres as a hard tissue implant material

As our lives are getting complex, there has been increasing dangerous situations such as car accident, industrial accident, intense outdoor sports, etc., causing bone fracture. Although our bone has self-healing ability, the healing rate can be significantly improved with the existence of implant material which is biocompatible and bioactive. This is because bioactive implant materials play an important role in guiding new bone tissue to ingrow and enhance regeneration rate. [1-3]

Ceramic microspheres have been widely used as a bone substitute because of many advantages of their shape and size. One advantage of using microsphere type is that their small size and spherical shape allow injection through a liquid medium, which leads to minimally invasive procedure. Second one is that uniform packing and homogeneous pore distribution built among spheres in defect site enhances the rate of new bone ingrowth. It is reported that pore size or spacing around 200 - 300μm is required for faster new bone tissue regeneration. [3-5]

Among many kinds of bioactive ceramics such as hydroxyapatite, various types of bioactive glass, calcium phosphates, hydroxyapatite (HA) has gained massive popularity in dentistry and orthopedics since it is similar to the inorganic part of natural bone. Bone mainly consists of inorganic hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) with low crystallinity and organic type I

collagen. HA is most stable phase of calcium phosphates which has 1.67 Ca/P ratio. It slowly provides Ca^{2+} and PO_4^{3-} ions in the body and accelerates activity of cells around it. [5-10]

1.2. Calcium phosphate bone cement

Calcium phosphate bone cement, invented by Brown and Chow in 1980s, has gained steady attention for a bone substitute because of its injectability and low temperature processing. Since its phase is transformed to low crystalline HA which is similar to natural bone, calcium phosphate bone cement exhibits great biocompatibility. [2, 9, 11]

Calcium phosphate bone cement system is basically composed of cement powder and hardening liquid. When they are mixed together, they become cement paste and undergo dissolution and precipitation. Then, the paste is quickly solidified and slowly transformed to apatite or brushite under humid condition. There are three types of calcium phosphate bone cement system. First one is single (mono) component system in which α-TCP is used as cement powder and hydrolysis of α-TCP is main reaction of this system. Second one is apatitic multi-components system in which TTCP and more acidic calcium phosphates set based on acid-base reaction. Third one is brushitic multi-components system in which β-TCP and an acidic component set based on acid-base reaction. Apatite formed from the first and second

system is stable under the physiological condition and has good biological properties. In contrast, brushite which is final product of the third system is metastable in our body. So, it is more biodegradable and less biocompatible than apatite.[2, 9, 12]

One of the greatest advantages of using calcium phosphate bone cement is that its room temperature processing breaks the limitation of incorporating drugs or proteins into ceramic materials. The limitation comes from heat treatment which is inevitable to achieve mechanical integrity of ceramic powders. However, for calcium phosphate cement, it is possible to mix ceramic powders and drugs before they get mechanical integrity because the cement is hardened through not thermal treatment, but dissolution and precipitation.[2, 4]

In particular, there is another excellent advantage of using apatitic bone cement, which is low crystalline HA as a final product. This is more favorable than high crystalline one as an implant material because high crystalline HA has low biodegradability, meaning that there needs to be second surgery. The final product of apatitic bone cement can be mainly resorbed by macrophages or osteoclasts since they drop the pH to the point where apatite is more soluble.[2]

1.3. The aim of this study

The aim of this study is to obtain an implant material with great efficiency, biocompatibility, and drug delivering capability, having advantages of both microspheres and bone cement. Therefore, microspheres were fabricated from bone cement, using water/oil emulsion method since bone cement is hydrophilic. Before bone cement paste becomes too hard, it was transferred to oil at a constant stirring speed. The paste made water/oil emulsion and was self-hardened within 10 min. Depending on the stirring speed, hardening liquid, type of oil, amount of surfactant, fabrication temperature, etc., the size and surface morphology was different. In this study, we used a certain condition to get appropriate size and morphology for implant particulates.

Chapter 2.

Enhancing Biocompatibility of Bone Cement Microspheres through Rapid Apatite Coating

2.1. Introduction

Microspheres from single component (α-TCP) bone cement are mechanically and chemically unstable because α-TCP powders are poorly interconnected and Ca/P ratio of α-TCP is 1.5(1.67 is most stable). So, the microspheres from α-TCP need to be converted to HA in SBF solution for around one week. In SBF solution, α-TCP dissolves and recrystallizes to calcium deficient hydroxyapatite (CDHA). The needle-like precipitated HA connects each α-TCP particles so that the microspheres gains mechanical stability. Although this process gives them mechanical and chemical stability, it requires too long incubation time for researchers. Therefore, in this study, we introduce a new efficient process which allows microspheres to have both stabilities in a very short period of time. TTCP and citric acid were added as bone cement powder and hardening liquid respectively, leading to higher mechanical property. Plus, instead of converting whole sphere into HA, the surface was modified by HA precipitation in a concentrated SBF like solution.[13-15]

2.2. Materials and methods

2.2.1. Fabrication of microspheres

Calcium phosphate cement powder consisted of α-TCP and TTCP at a molar ratio of 2:3. α-TCP was obtained by sintering at 1300℃ for 6hours and quenching the mixture of 54 g (0.73 mol) of DCPA($CaHPO_4$, Sigma-Aldrich C7263) and 20 g (0.2 mol) of calcium carbonate($CaCO_3$, Sigma-Aldrich C4830). TTCP was obtained by sintering at 1500℃ for 6hours and quenching the mixture of 136.06 g (1 mol) of DCPA and 90.081 g (0.9 mol) of calcium carbonate. α-TCP and TTCP were milled in a planetary mill and sieved to obtain powder smaller than 37μm. The hardening liquid was prepared by dissolving 10wt% of citric acid into 1M of Na_2HPO_4 solution. The cement paste was produced by mixing the powder and the hardening liquid at 0.5L/P (ml/g) ratio for 30 seconds. Subsequently, the paste was emulsified by stirring at 600rpm for 10minitues in the olive oil at 10℃ containing 10 vol. % of the Labrafil®(1944 CS, Gattefossé, France) as surfactant. Separated from the oil, microspheres were washed several times with acetone and ethanol and air dried then sieved to get the uniform size of microspheres ranged from 180μm to 250 μm.

2.2.2 Rapid Apatite Coating

10mg of microspheres were immersed in 10ml of 10x SBF-like

solution. SBF is known to be a very good coating solution due to low temperature processing, bone-like apatite product, and even deposition on complex geometry. To prevent aggregation of spheres during the immersing process, the container kept rotating in a ball milling machine for 2h at 40rpm. Then the microspheres were washed several times with deionized water and dried at 37℃. [16, 17]

2.2.3 Characterization

Samples were sputter coated with platinum and then their morphology was observed by scanning electron microscopy (SEM; JSM-6360, JEOL, Japan).

The composition of the samples was investigated with X-ray diffractometer (XRD, D8-advance, Bruker Co., Germany) with Cu-Kα radiation from 25 to 35 (2θ) at a scan rate of 2˚/min.

A hardness test was performed to study the mechanical properties of the samples, using the Vickers hardness tester (HV-114, Mitutoyo, Japan). To prepare the specimen for the test, the cross section of the microspheres (355um-500um) was disclosed after grinding 250um of the epoxy which was filled with the microspheres as shown in Figure 5. Then, a pyramid shaped diamond indenter pressed it with the load of 1kgf and duration time of 15seconds. The Vickers hardness was calculated by the formula shown below.

$$H_v = \frac{1.854 \times 9.807 \times F}{D^2} (MPa)$$

Where: F = Load in kg

D = Arithmetic mean of the two diagonals, d_1 and d_2 in mm

2.2.4. *In vitro* biological analysis

Three kinds of samples (alpha-stabilized, composite-stabilized, and composite-coated) were prepared to evaluate biological properties by in vitro test using MC3T3-E1 cells (ATCC, CRL-2593; Rockville, MD). For the cell attachment test, 7Φ in mm carbon tape was attached on Thermanox® plastic coverslips (174950, NUNC™, USA). The microspheres are poured onto the carbon tape, making one layer of the spheres. Then, the plastic coverslips with microspheres was transferred into 4well plate. For the cell proliferation test, carbon tape was fully attached at the bottom of the 96well plate and one microspheres layer is made by pouring them on the tapes. MC3T3-E1 cells were seeded on each samples at a density of $0.7x10^4$ cells/ml and cultured in a Minimum Essential Medium (α-MEM, Welgene Co, Ltd., Korea) containing 5% Fetal Bovine Serum (FBS, Life Technologies, Inc., USA) and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, GIBCO, Grand Island, NY) in a humidified incubator with 5% CO_2 at 37 ℃. After 3daysof culturing, the morphology of cells was observed using scanning electron microscopy.Prior to the observation, the cells were fixed with 2.5%

glutaraldehyde for 10 min followed by dehydration in graded ethanol (70, 95, and 100%). The samples were immersed in hexamethyldisilazane for 10 min then air dried. After 3 days, 7 days, and 10 days of culturing, cell viability was examined using MTS assay (CellTiter 96 Aqueous One Solution, Promega, USA).

2.2.5. Statistical analysis

The data are presented as the mean ±SE of mean. Statistical analysis was performed using a one-way analysis of variance (ANOVA) and p values less than 0.01 were considered to be statistically significant.

2.3. Results and discussion

2.3.1. Microstructure and Mechanical property

As illustrated in Figure1, microspheres were fabricated with multi-component system. Emulsified bone cement paste was self-set as spherical shape in the oil, and then washed. The morphology of sieved microspheres was shown in Figure 2. When they were in aqueous solution, the set microspheres slowly become harder, being transformed to HA. The reactions and microstructure change were presented in Figure 3. When converted to HA, microspheres had needle-like microstructure as shown in Figure 3(B).

To analyze the mechanical property, microstructure was investigated. Two kinds of spheres had different microstructure. α-TCP microspheres seemed to have low strength because there were undissolved and poorly packed α-TCP particles in the cross section as shown in Figure 4. On the other hands, in composite microspheres, the spacing among α-TCP particles was well filled with recrystallized TTCP. Since in the physiological condition at pH 7.4, TTCP dissolves much quicker than α-TCP, dissolved TTCP quickly occupied the space and then recrystallized. [18]

To measure the mechanical property of the two kinds of system, single component (α-TCP) and multi-component (composite of α-TCP and TTCP at a molar ratio of 2:3), Vickers hardness test was performed. Figure 6 shows that hardness of multi-component was higher by about 40% than single component in bulk form and by 25% in microsphere form. This increment stemmed from the microstructure of each system depending on existence of TTCP and citrate ions. Chelation of citrate ions from citric acid improves the dispersion of calcium ions in the cement paste, resulting in homogeneous microstructure. [19-21]

2.3.3. Coating layer analysis

Although quick dissolution rate of TTCP played an important role in improvement of the mechanical property, this negatively influenced inner HA formation because TTCP dissolved away rather than participated in HA formation with α-TCP.

In order to obtain bonelike apatitic surface within a short period of time, a calcium phosphate solution with very high ionic strength (>1100 mM) was utilized. The composite microspheres were immersed in 10x SBF-like solution for 2hours at 37℃ and at constant rotation speed of 40rpm. Ionic concentrations of 10x SBF-like solution and SBF solution were compared in table1.[16]

In the 10x SBF-like solution, precipitation kinetics and precipitate structure are affected by NaCl and HCO_3^- contents. Compared to normal SBF solution, the 10x SBF-like solution contains more NaCl by around seven times and less HCO_3^- by one third. NaCl increases ionic strength, leading to delaying Ca/P precipitation in the solution. So, more precipitation favorably occurs not in the solution but on the surface of the microspheres. HCO_3^- is known to reduce apatitic growth rate. So, less concentration of HCO_3^- boosts apatite formation. [22]

In Figure 7(A), without coating, the surface of microspheres looked quite smooth. As the coating time increased, the coating layer got thicker and apatite crystal size became bigger. After 30mins of coating, about 1μm of apatitic coating layer was formed (Figure 7(B)). It reached to 2.5μm with addition of 1hour and half in Figure 7(C). For further coating time, there was not noticeable increase in the thickness. That is because the concentration of calcium and phosphate ions was decreased and it did not have much driving force compared to the initial state. So, the reaction was going to be saturated. For the thicker coating layer, medium refreshment should be necessary.

In addition, the surface roughness was controllable with the solution amount. Since osteoblast cells prefer rough surface to smooth one, it is important for implant materials to have rough surface. Figure 8 presented that the amount of solution compared to that of sphere determined the surface morphology. When solution/sphere in ml/mg increased, the surface of the

microsphere became rough with a fixed coating time. This is because apatite deposition was able to be active for longer period of time with abundant Ca/P ions in the solution. When solution/sphere decreased, the surface became smoother with a fixed coating time. This is because apatite deposition reaction was rapidly slowed down as loosing Ca/P ions and driving force quicker in the solution. [23]

2.3.4. Phase transformation

XRD analysis was performed to check how the HA coating layer is protective from loosing TTCP and how much HA is formed and reactants is remained in the physiological condition up to 2 weeks. After one week without HA coating layer, most α-TCP was still remained but no TTCP. There was little difference in the amount of reactants and HA after 2 weeks as shown in Figure 9(A). Consequently, the reaction of α-TCP and TTCP was not in balance in microspheres without HA coating layer. On the other hands, microspheres with HA coating layer already consumed some reactants and produced some HA in 10x SBF-like solution for a few hours. After one week in SBF solution, TTCP was remained and plenty of α-TCP was consumed, producing equivalent HA. Surprisingly, even after 2weeks, there was still TTCP and α-TCP kept participating in the reaction and produced more HA as shown in Figure 9(B). Therefore, the final product of microspheres without

HA coating layer was mostly unreacted α-TCP and some low crystalline HA, and the final product of microspheres with HA coating layer was a little bit of β-TCP and mostly low crystalline HA. The latter can be thought to exhibit greater biocompatibility since it consists of mostly low crystalline HA and a small quantity of β-TCP, which can be considered more biocompatible and bioactive than HA alone. [9]

2.3.5. In vitro biological analysis

To measure the biological property of rapidly formed apatitic coating layer, three kinds of samples were prepared. First one is α-TCP microspheres immersed in normal SBF solution for 1 week. In Figure 10(A), α-TCP microspheres formed apatitic surface in normal SBF solution since α-TCP has a good hydrolysis property. Second one is composite (α-TCP: TTCP = 2:3) microspheres immersed in normal SBF solution for 1week. In Figure 10(B), this system did not form typical apatitic surface in normal SBF solution because there was relatively less amount of α-TCP. Third one is composite microspheres coated in 10x SBF-like solution for 2 hours. In Figure 10(C), this solution helped to make the apatitic layer on the composite microspheres within only hours of coating time.

In order to measure initial attachment of pre-osteoblast cells on

microspheres, MC3T3-E1 cells were cultured on them. After 3days of culturing, cells were well spread on three types of microspheres as shown in Figure 10(D)(E)(F). This means that all the samples were well washed from the oil and there were no toxic component. It looked like cells on the coated microspheres were spread more and proliferated more.

To obtain a quantitative biological property, cell proliferation was measured by MTS assay (Figure 11). As seen in the cell adhesion test, cells grew well on all type of samples. The number of cells was slightly higher on composite-coated microspheres at day 3. This gap was getting bigger as time goes by. Then it made a significant difference at day 10. The result shows that the apatitic coating layer formed by 10x SBF-like solution played an important role in cell viability since it regulated dissolution rate of reactant and made more stable surface. The first and second specimen contains much α-TCP in their phase which is unstable material in physiological condition. Even though α-TCP and TTCP are suppliers of original substances for bone formation, too much amount of release adversely influenced on cells. For further study, cells behavior will be investigated depending on the morphology or thickness of the coating layer. [24]

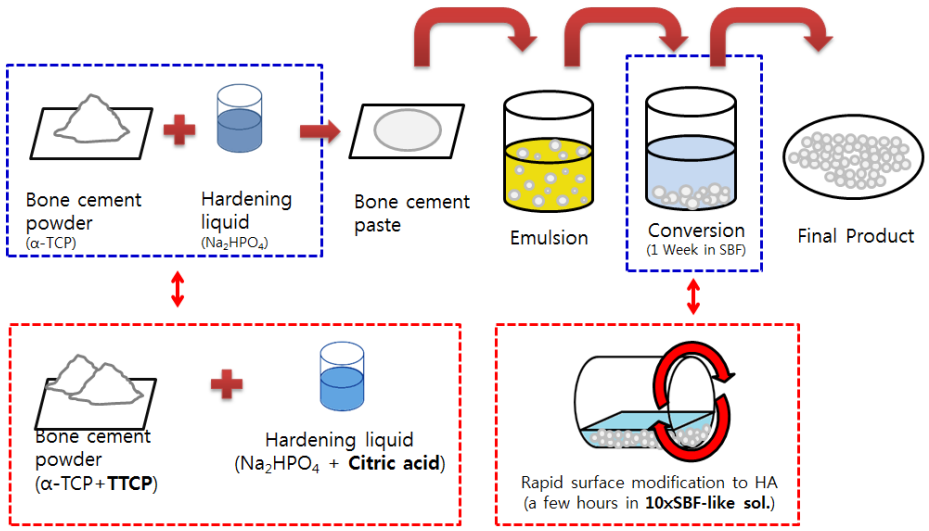

Figure 1.Schematic diagram of general (blue box) and new (red box) fabrication methods.

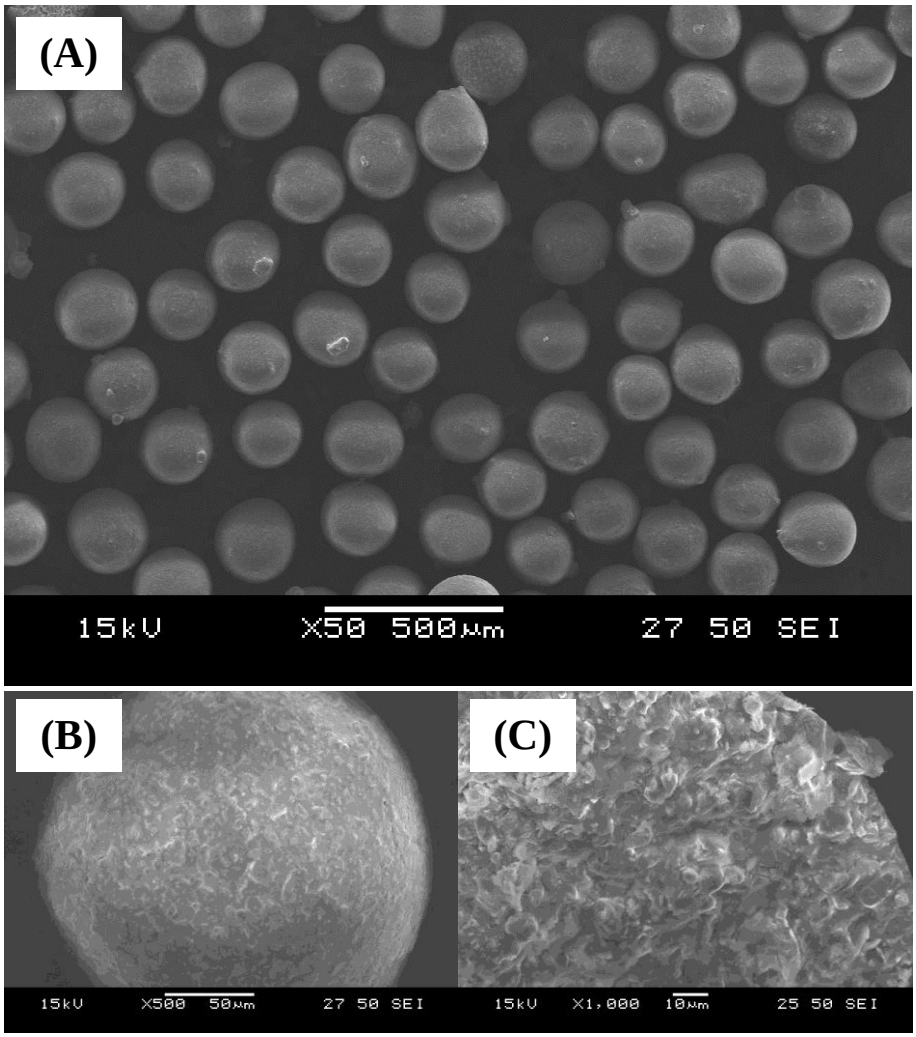

Figure 2. Scanning electron microscopy (SEM) images of (A) bone cement microspheres (180-250μm) and their higher magnification of (B) surface and (C) cross section.

Self-setting stage (very rapid)

$$\mathbf{2Ca_3(PO_4)_2} + 2H_3PO_4 + 12H_2O \rightarrow 6Ca(HPO_4)\cdot 2H_2O$$

α-TCP *Phosphoric acid* *Water* *DCPD*

$$\mathbf{Ca_4(PO_4)_2O} + 2H_3PO_4 + 7H_2O \rightarrow 4Ca(HPO_4)\cdot 2H_2O$$

TTCP *Phosphoric acid* *Water* *DCPD*

Hardening stage (slow)

$$\mathbf{2Ca_4(PO_4)_2O} + 2Ca(HPO_4)\cdot 2H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 4H_2O$$

TTCP *DCPD* *Hydroxyapatite* *Water*

$$\mathbf{2Ca_3(PO_4)_2} + \mathbf{Ca_4(PO_4)_2O} + H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2$$

α-TCP *TTCP* *Water* *Hydroxyapatite*

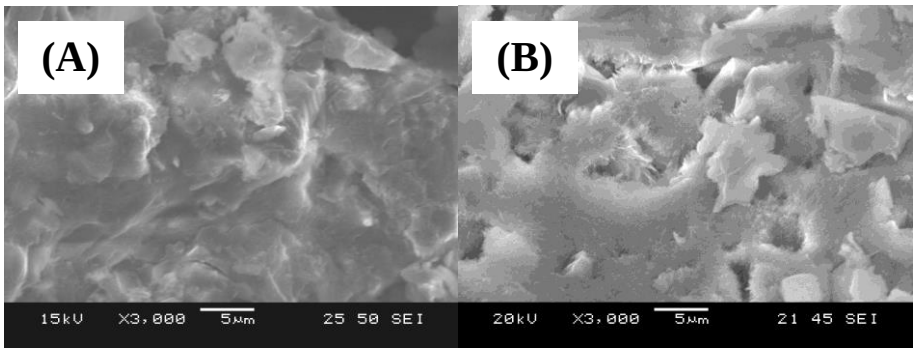

Figure 3. Chemical reactions mainly occurring in the microspheres and the microstructure after (A) self-setting and (B) hardening stage.

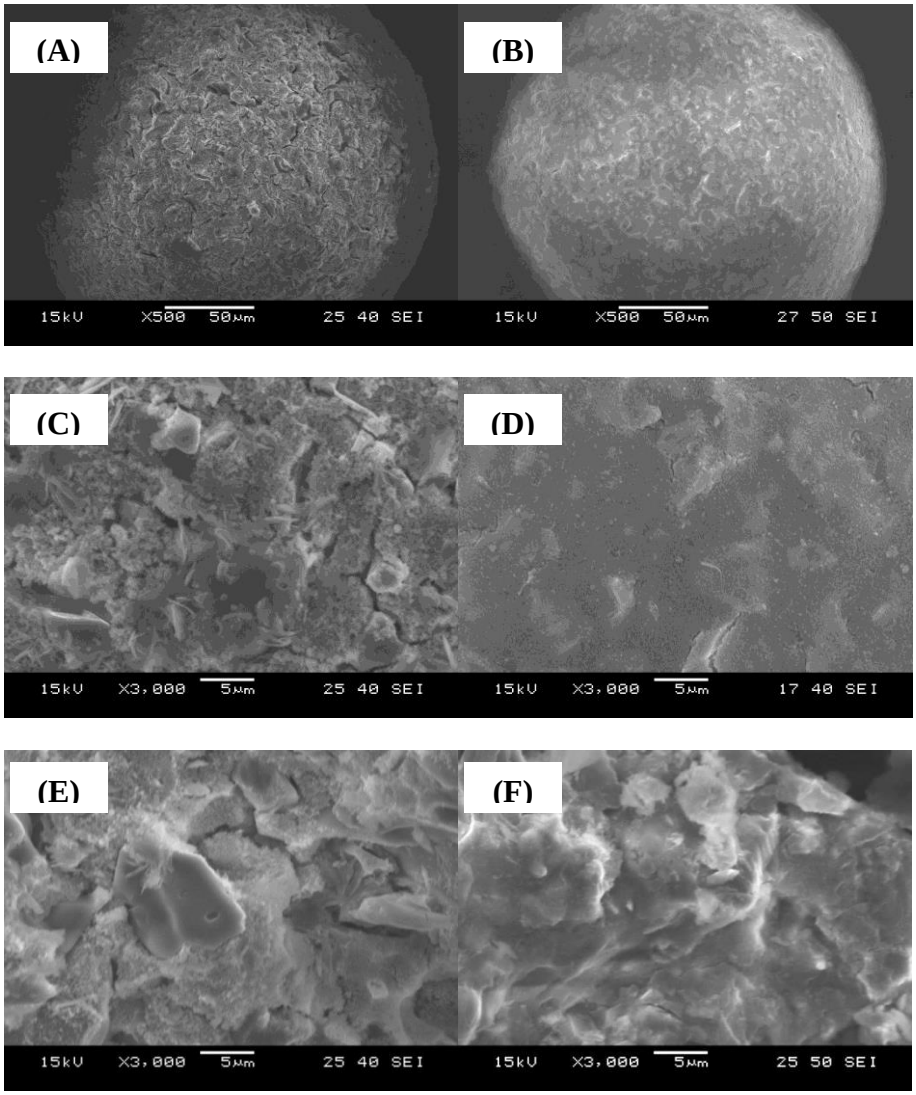

Figure 4. SEM images of (A) α-TCP microspheres (left) and (B) composite microspheres (right), and higher magnification of (C)(D) surface (middle) and (E)(F) cross section(bottom).

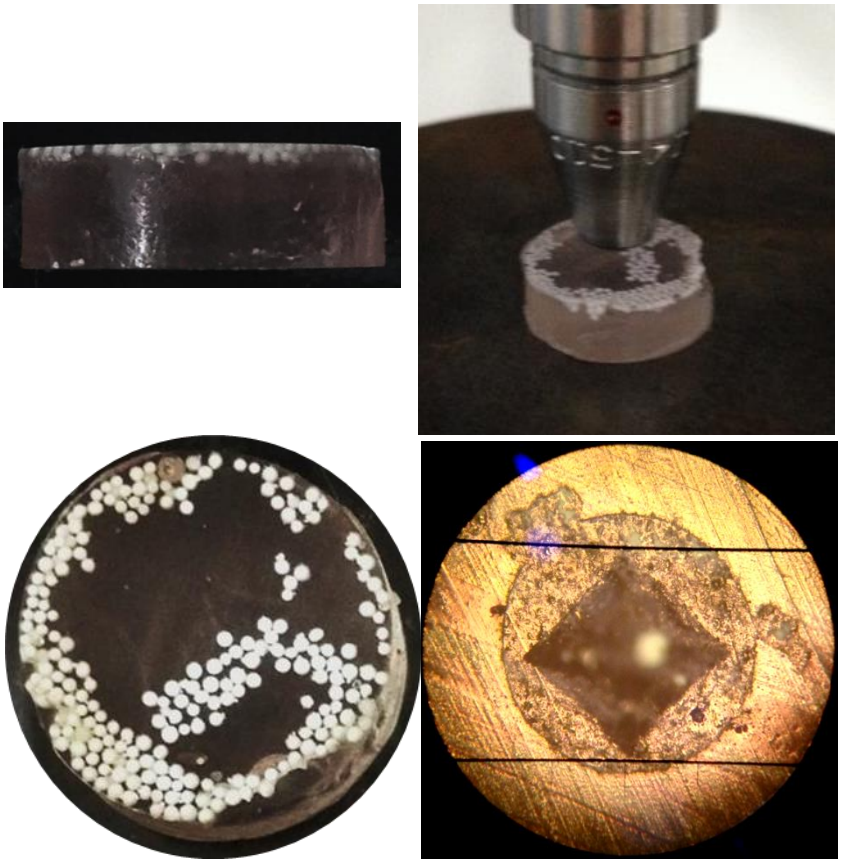

Figure 5. Optical images of specimen for Vickers hardness test of microspheres.

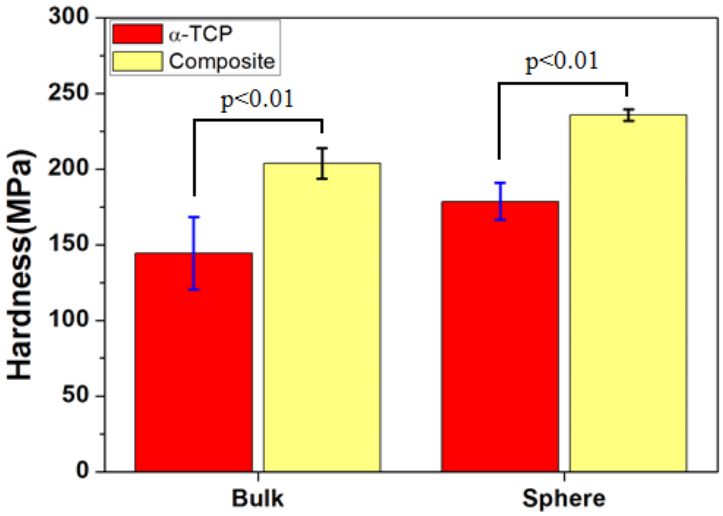

Figure 6. Vickers hardness of α-TCP and composite with respect to their form (bulk and microsphere) after complete setting. (n=5)

	Na^{+}	K^{+}	Ca^{2+}	Mg^{2+}	Cl^{-}	HPO_4^{2-}	HCO_3^{-}
SBF	142	3.6-5.5	**2.1-2.6**	1	95-107	**0.7-1.5**	27
10x SBF-like solution	1020	5	**25**	5	1065	**10**	10

Table1. The ionic concentration (mM) of SBF solution and 10xSBF-like solution.

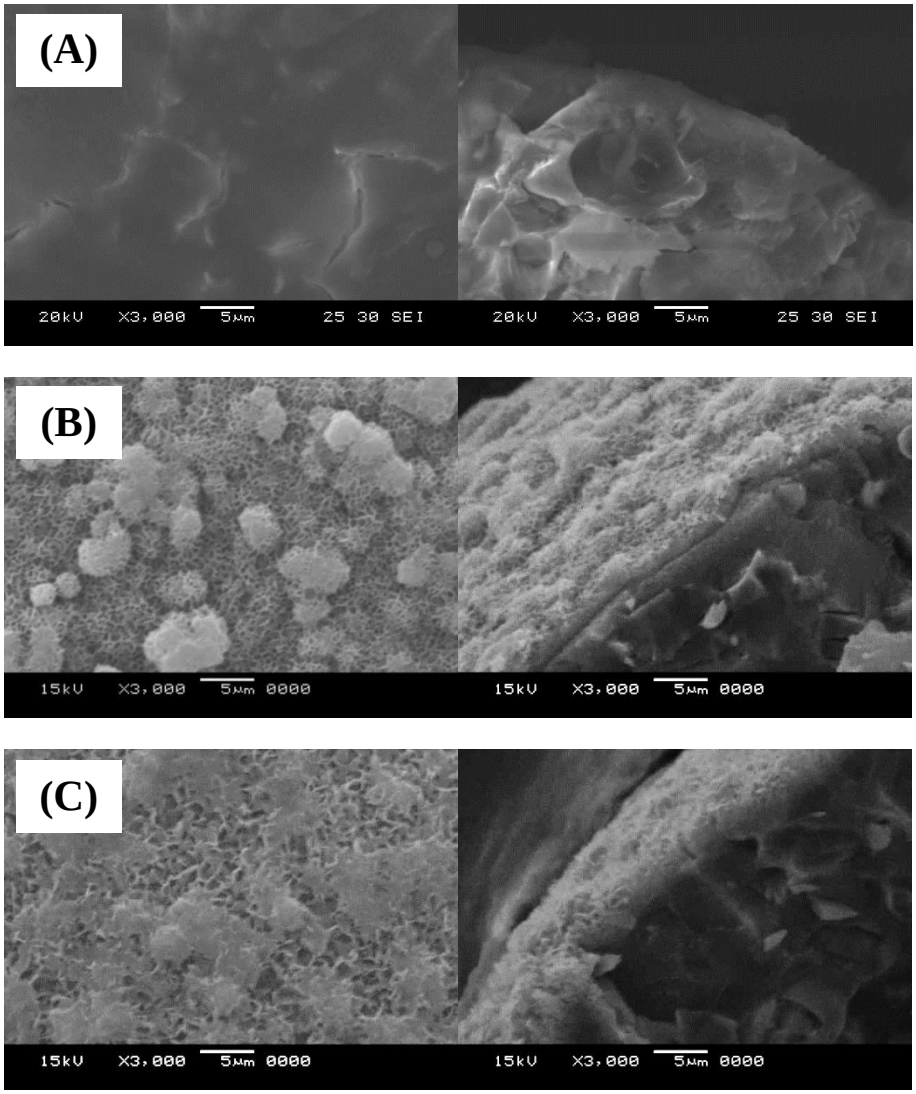

Figure 7. SEM images of surface (left) and cross sectional (right) view of (A) bare and coated ((B) 0.5h and (C) 2h) microspheres

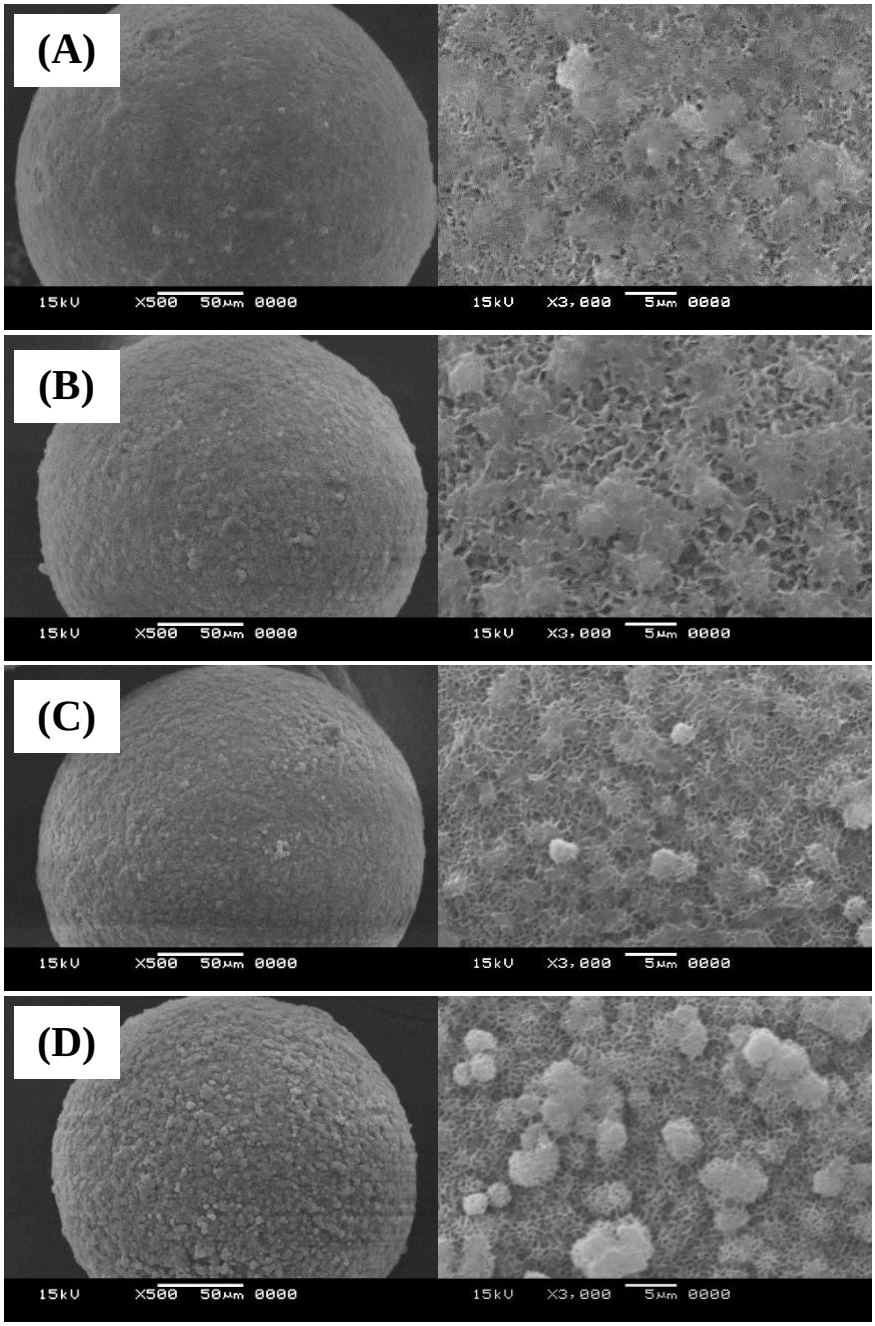

Figure 8. SEM images of coated microspheres with different coating time and solution/sphere ratio((A) 2h and 0.5ml/mg, (B) 2h and 1ml/mg, (C) 0.5h and 0.5ml/mg, and (D) 0.5h and 1ml/mg)

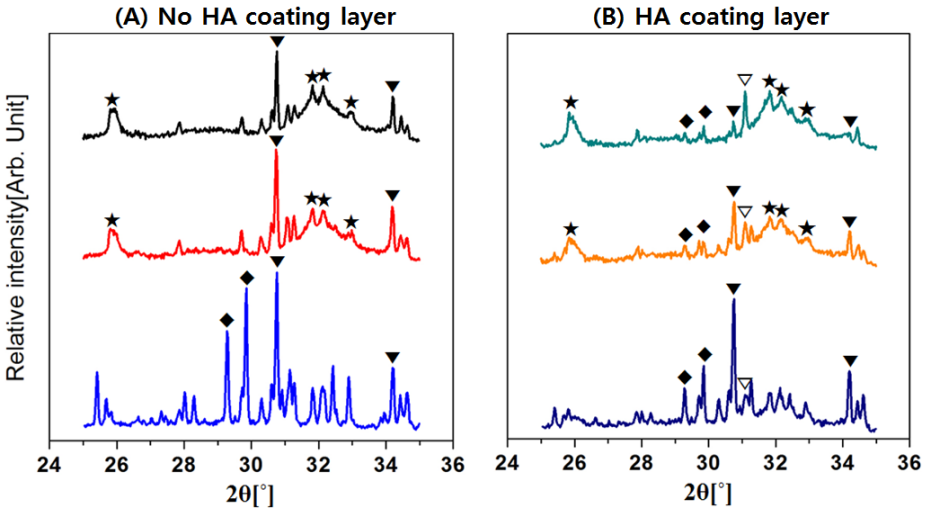

Figure 9.XRD patterns of microspheres with (A) no HA coating layer and (B) HA coating layer before(bottom) and after the SBF test for 1 week(middle) and 2 weeks(top). (▼: α-TCP, ▽: β-TCP, ◆: TTCP, ★: HA)

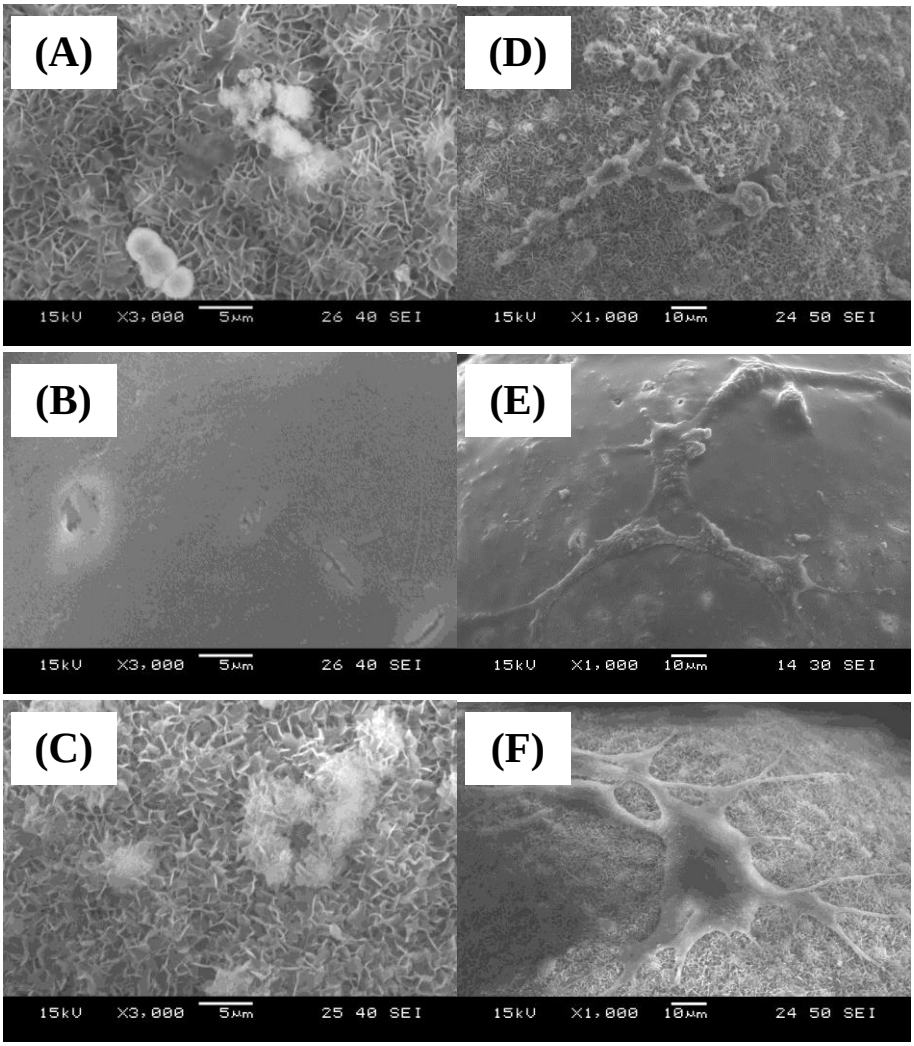

Figure 10.SEM images of the surface of (A) alpha-stabilized, (B) composite-stabilized, and (C) composite-coated microspheres and MC3T3-E1 cells after 3days culturing on (D) alpha-stabilized, (E) composite-stabilized, and (F) composite-coated microspheres.

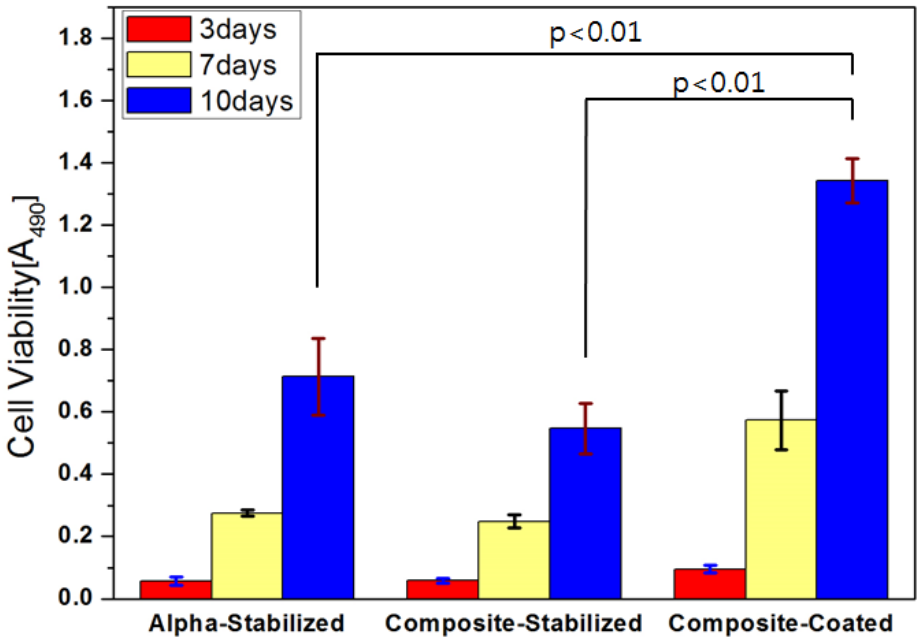

Figure 11. Cell viability at 3, 7, 10 days measured by MTS assay on alpha-stabilized, composite-stabilized, composite-coated microspheres. (n=3)

Chapter 3.

Sustained Release of Biological Molecules from Hydroxyapatite Microspheres

3.1. Introduction

Bioactive ceramics are often incorporated with drugs or bioactive proteins to accelerate bone healing rate. However, heat treatment in ceramic processing has limited bioceramics for drug delivery applications. For example, most heat treatment-requiring ceramics did not have choice but immersing samples in a drug solution after fabrication since biological molecules are easily denatured at elevated temperature. Obviously, most biological molecules are coated on the ceramic surface and released up in a short period of time.[3, 13, 25]

During the bone cement microspheres processing, drugs can be mixed with bone cement powders so that drugs are well spread in side of the microspheres and can be released for a longer period of time. However, previously developed bone cement microspheres using α-TCP needs to be stabilized in SBF solution to increase biocompatibility and mechanical property, leading to lots of drug loss. Therefore, this study introduced a new method to give microspheres mechanical and chemical stability without drug loss. [13, 14]

3.2. Materials and method

3.2.1. Fabrication of protein loaded microspheres

Calcium phosphate cement powder consisted of α-TCP and TTCP at a molar ratio of 2:3. α-TCP was obtained by sintering at 1300℃ for 6hours and quenching the mixture of 54 g (0.73 mol) of DCPA($CaHPO_4$, Sigma-Aldrich C7263) and 20 g (0.2 mol) of calcium carbonate($CaCO_3$, Sigma-Aldrich C4830). TTCP was obtained by sintering at 1500℃ for 6 hours and quenching the mixture of 136.06 g (1 mol) of DCPA and 90.081 g (0.9 mol) of calcium carbonate. α-TCP and TTCP were milled in a planetary mill and sieved to obtain powder smaller than 37μm. The hardening liquid was prepared by dissolving 13.2wt% of citric acid into 1.32M of Na_2HPO_4 solution. When the cement power was mixed with the hardening liquid for 30 seconds, GFP solution of 0.3mg/ml was also added, taking 25% of whole liquid phase. Then, the concentration of citric acid and Na_2HPO_4 became 10wt% and 1M respectively. Subsequently, the paste was emulsified by stirring at 600rpm for 10minitues in the olive oil at 10℃ containing 10 vol. % of the Labrafil® (1944 CS, Gattefossé, France) as surfactant. The oil containing microspheres was transferred to a glass vial and kept in 37℃ oven. After 3days, microspheres were separated from the oil and washed several times with acetone and ethanol and air dried then sieved to get the uniform size of microspheres ranged from 180μm to 250 μm. For *in vivo* animal test,

bone morphogenetic protein (rhBMP-2) of 0.3mg/ml was loaded with the same method.

3.2.2. Characterization

Samples were sputter coated with platinum and then their morphology was observed by field emission scanning electron microscopy (FE-SEM;SUPRA 55 VP, CARL ZEISS, Germany).

The composition of the samples was investigated with X-ray diffractometer (XRD, D8-advance, Bruker Co., Germany) with Cu-Kα radiation from 25 to 35 (2θ) at a scan rate of 2˚/min.

3.2.3. Green fluorescent protein release test

Instead of using drugs, GFP was utilized since it is also possible to visualize how the molecules were distributed. 20mg of GFP loaded microspheres were immersed in a glass vial which contains 1.5ml of Phosphate buffered saline (PBS). The vial was kept at 37℃for 28days. The medium was refreshed at every test. GFP was observed by confocal laser scanning spectroscopy (CLSM, Zeiss-LSM510, Carl Zeiss Inc., NY, USA).The concentration of GFP released was determined by UV spectrophotometer (UV-1700, Shimadzu, Japan). Absorbance values measured at 220 nm was converted to the concentration of the GFP using the linear

relationship between optical absorbance (x) and GFP concentration (y), which is y = 1.2783x with R^2=0.9999.

3.2.4. Preliminary *in vivo* animal test

The in vivo animal test was carried out on a female rat with two kinds of microspheres (with BMP and without BMP). A combination of 0.07ml of 2% Xylazine HCL (Rompun, Bayer Korea, Korea) and 0.14ml of Tiletamine HCL (Zoletil, Virbac lab, France) as the general anesthesia and Lidocaine (Yuhan Corporation, Korea) with 1:100,000 epinephrine as the local anesthesia was injected. Bilateral full-thickness defects 5 mm in diameter were created in parietal bone using an electric drill. The calvarial defects were implanted with 2 kinds of microspheres (with BMP and without BMP). After surgery, the wounds were sutured with Surgisorb (Samyang Ltd, Korea) and cephradine (Bayer Korea, Korea), antibiotic, was injected for 2 days.

After four weeks of healing, the rat was sacrificed to extract their bone defect regions. The harvested bone tissues were scanned by micro-CT to evaluate new bone formation and bone ingrowth.

For histological evaluation, the extraction regions were fixed in a neutral 10% formaldehyde solution, followed by decalcification in a low concentration of hydrochloric acid solution, and blocks were made using

paraffin. 5µm paraffin sections were prepared and stained with the Goldner's trichrome. The microscopic images of the stained sections were obtained using Axioskop microscopy (Olympus BX51, Olympus Inc., Japan).

3.2.4. Statistical analysis

The data are presented as the mean ±SE of mean.

3.3 Results and discussion

3.3.1. Phase transformation

It is important for the solidified microspheres to avoid direct contact to hydrophilic medium since there could be loss of drugs. Previously, bone cement microspheres must be immersed in SBF solution for 1week to be completely set. With this method, large amount of drug could be lost. Therefore, the solidified microspheres were kept isolated in the oil for several days, preventing drugs from diffusing into medium (Figure 12). [14]

With different isolation time in olive oil, the phase of microspheres was analyzed in Figure 13. In the oil, moisture from hardening liquid is trapped in microspheres and participates in the phase transformation reaction. Only after 1 day, large amount of hydroxyapatite was observed. The broad range peaks indicate the HA has poor crystalline structure, which is more preferable to high crystalline due to better biodegradation. Phase transformation kept going and the reaction was continuous at day 7 but almost saturated at day 3. So, 3days of isolation was chosen for this study.

3.3.2. Sustained release – GFP

GFP was coated on the microspheres by immersing them in GFP solution for 4 hours to see how fast it could be released from the surface of the microspheres. And for sustained release, GFP solution was initially mixed

with bone cement powder in making cement paste. CLSM images of the cross section in Figure 14 showed GFP was loaded well as we expected. For GFP-coating sample, most GFP was on the surface and for GFP-mixing sample, GFP was well distributed in the sphere. Interestingly, GFP molecules were concentrated in the pore walls where HA needle must be. This is because nano HA needle provided very high surface area for much more protein molecules to be bound.

Both kinds of samples were immersed in PBS solution for one month to see how much GFP could be still in there. One month of release test was performed and the result was shown in Figure 15. After one month of release test, most coated GFP was released and hardly seen, but mixed GFP was still inside of microspheres since HA has high binding affinity to proteins.[2, 5]

To investigate more accurate release pattern, the concentration of released GFP was observed by UV-spectrophotometer, using standard curve in Figure 16. In this test, most coated GFP was released in 10 days, but mixed GFP was continuously released from microspheres even after one month (Figure 17), suggesting potential incorporation of drugs or growth factors.

3.3.3. Bone regeneration

To confirm the effect of BMP released from the microspheres, two kinds of samples were implanted in a rat calvarial defect (with BMP and

without BMP) as shown in Figure 18. The rat was sacrificed. after 4 weeks of implantation. When the defect was opened, implanted microspheres were covered by numerous soft tissues.

Micro CT images in Figure 19 showed that both kinds of microspheres do not have infectious problems and have osteoconductivity owing to HA. [2, 5, 11] As TTCP based bone cement is known to be biocompatible and osteoconductive in rat calvarial defect, new bone was well grown from the boundary of the defect to the center. In the defect area, everything but spherical objects seemed to be new bone, which was noticeable in the cross section of both samples. In the middle of the defect, large new bone is seen with the existence of BMP since osteoinductivity of BMP accelerated new bone formation without host bone. [2, 26, 27]

3-D images of microspheres with BMP extracted from rat calvarial defect after 4 weeks of implantation were constructed to clearly observe newly formed bone as seen in Figure 20. On top view, two large new bone pieces were observed, which are light-green in the middle of the defect. At bottom view, the shape of defect was distorted from a circle due to new bone grown from the defect boundary.

Figure 21 (A)-(D) show representative histological corss-sectional images of the specimen after four weeks of healing. Osteoconduction occurred from defect sites and extended toward the space built among spheres (Figure 21(B)). New bone formation was also occurred even in the inner parts

of the defect (Figure 21(C)-(D)), showing the great bone regeneration ability of the BMP-loaded microspheres.

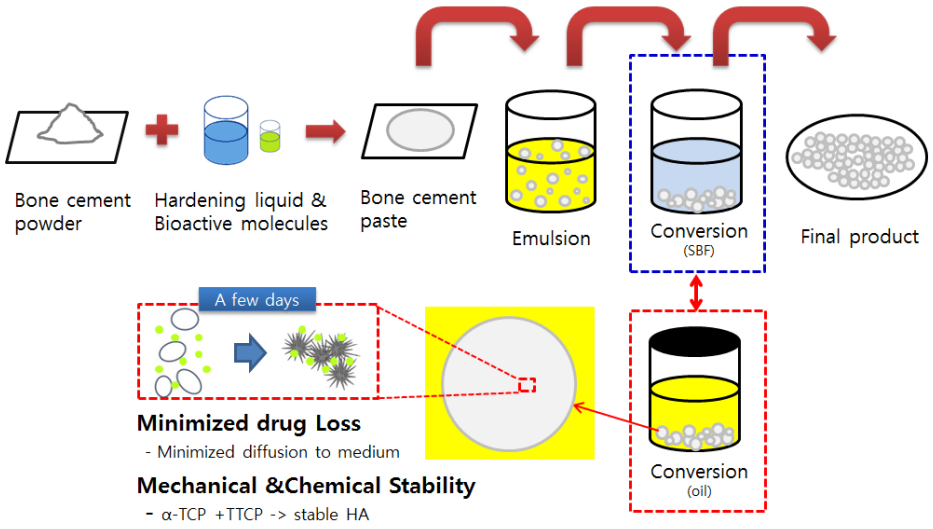

Figure 12. Schematic diagram of existing (blue box) and new (red box) fabrication methods of drug loaded microspheres

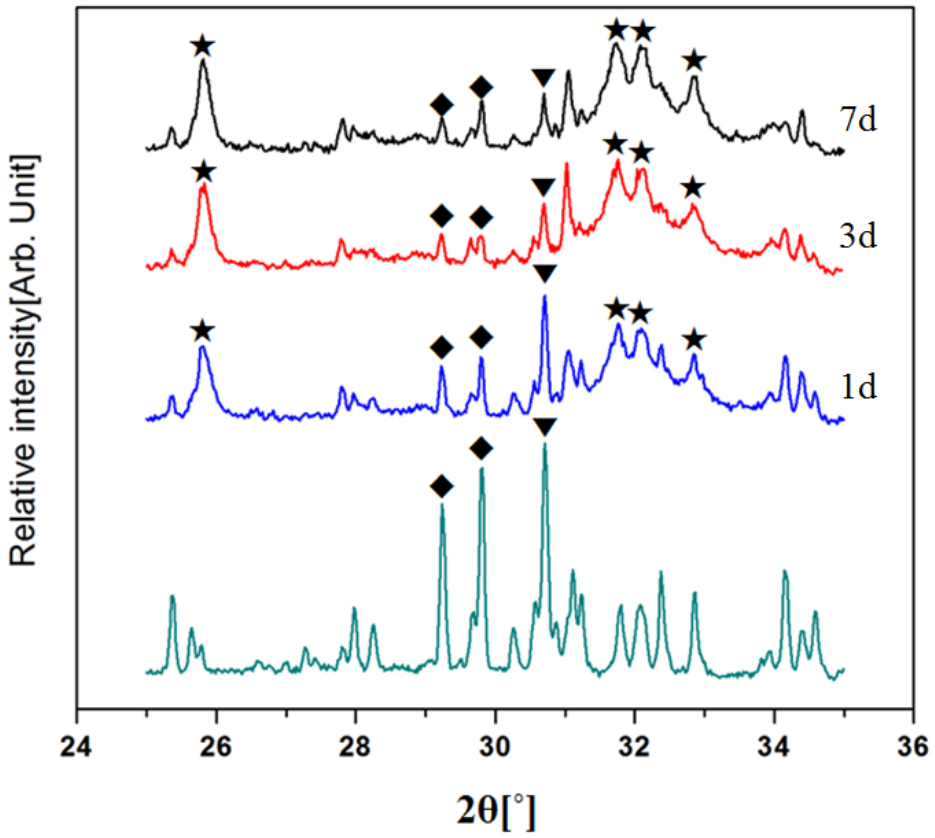

Figure 13. XRD patterns of microspheres isolated in oil for up to 7days.

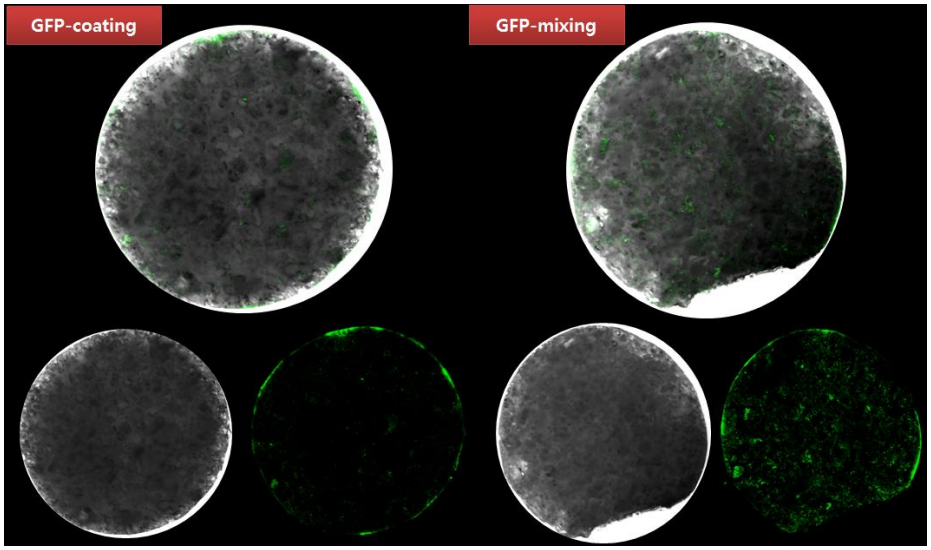

Figure 14. Confocal laser scanning microscopy (CLSM) images of the cross section of GFP-coating and GFP-mixing microspheres

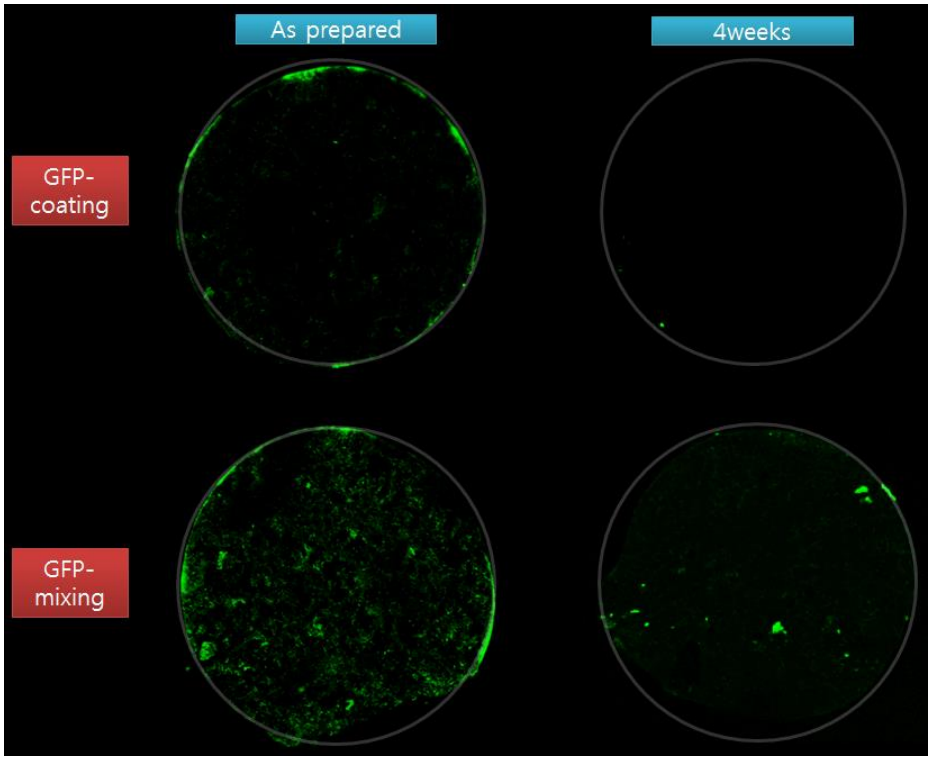

Figure 15. Confocal laser scanning microscopy (CLSM) images of the cross section of GFP-coating and GFP-mixing microspheres before and after release for 4 weeks.

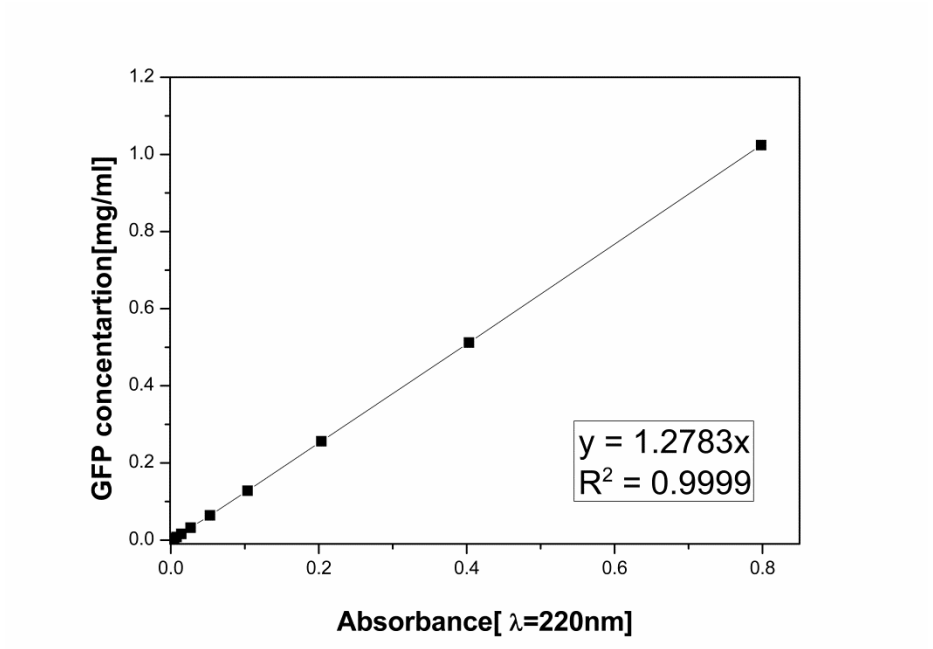

Figure 16.Standard curve for conversion from optical density to GFP concentration.

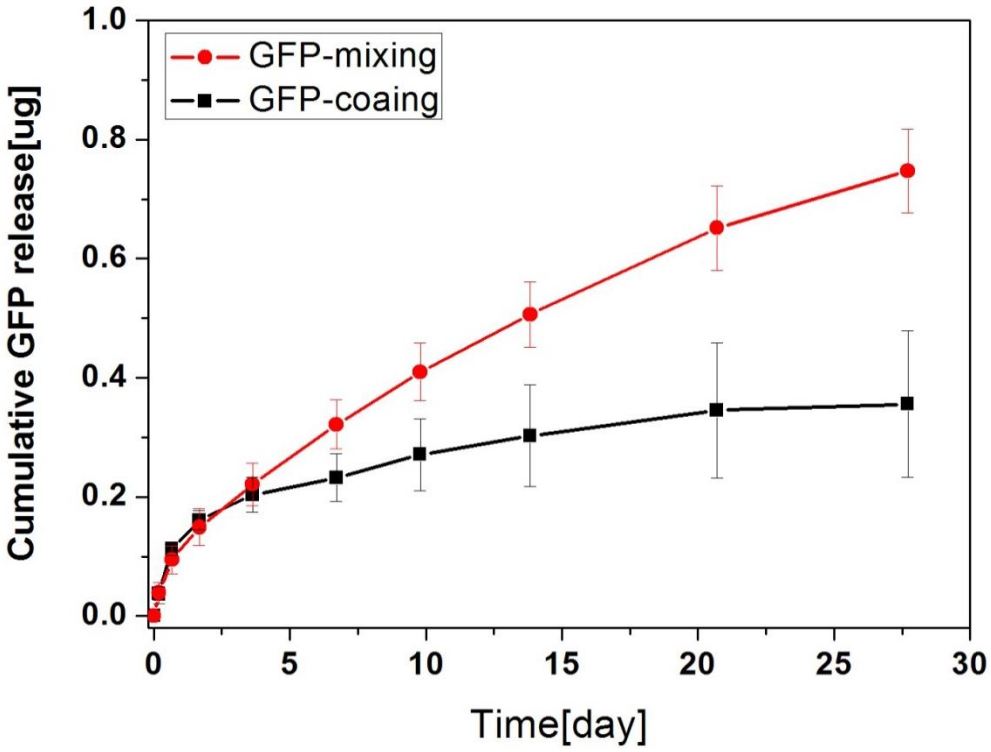

Figure 17.Cumulative GFP release from microspheres of GFP-mixing and GFP-coating.

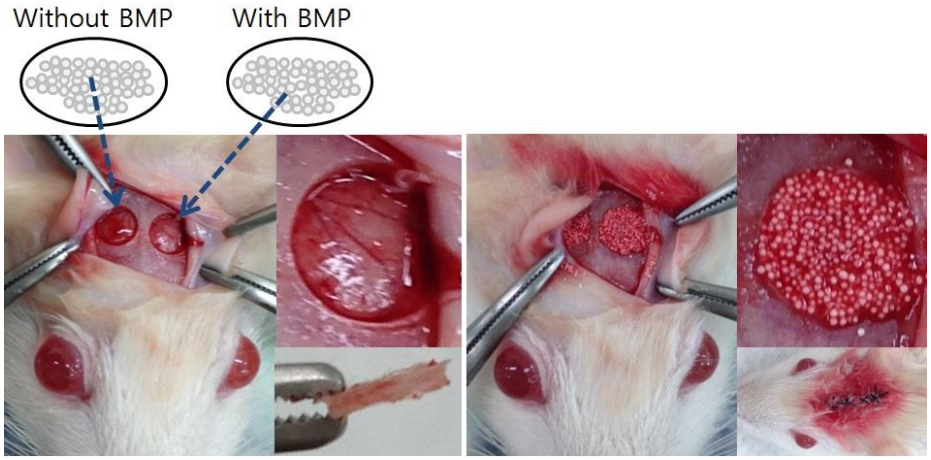

Figure 18. Optical images of implantation procedure on rat calvarial defects.

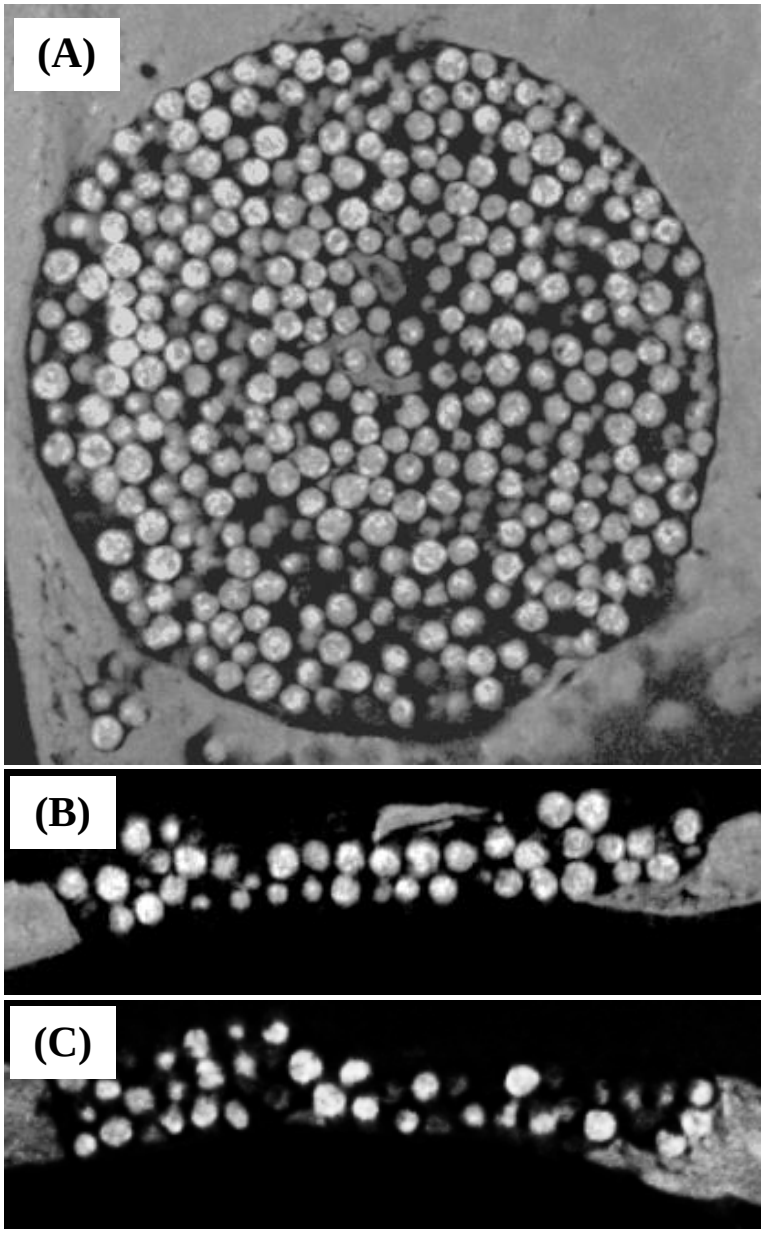

Figure 19. (A) Top view of micro CT images of microspheres with BMP and cross sectional view of microspheres (B) with BMP and (C) with BMP extracted from the rat calvaria 4weeks after implantation.

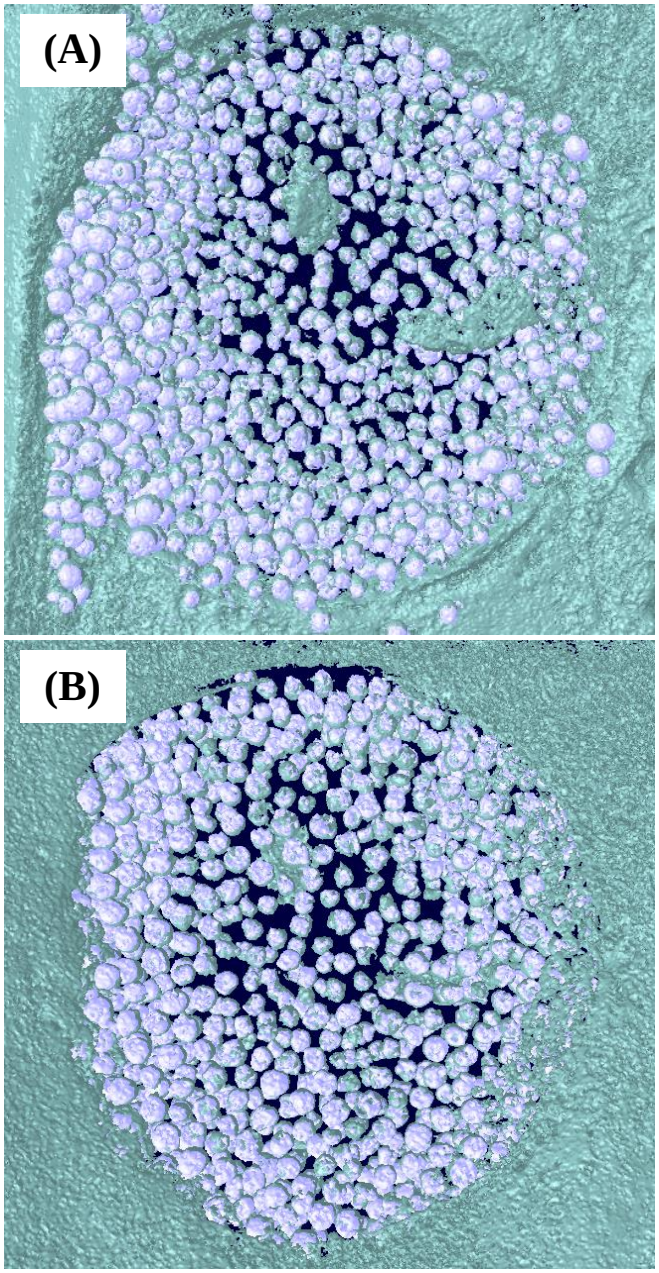

Figure 20. (A) Top and (B) bottom view of 3-dimentional images of microspheres with BMP extracted from the rat calvarial defect after 4weeks of implantation

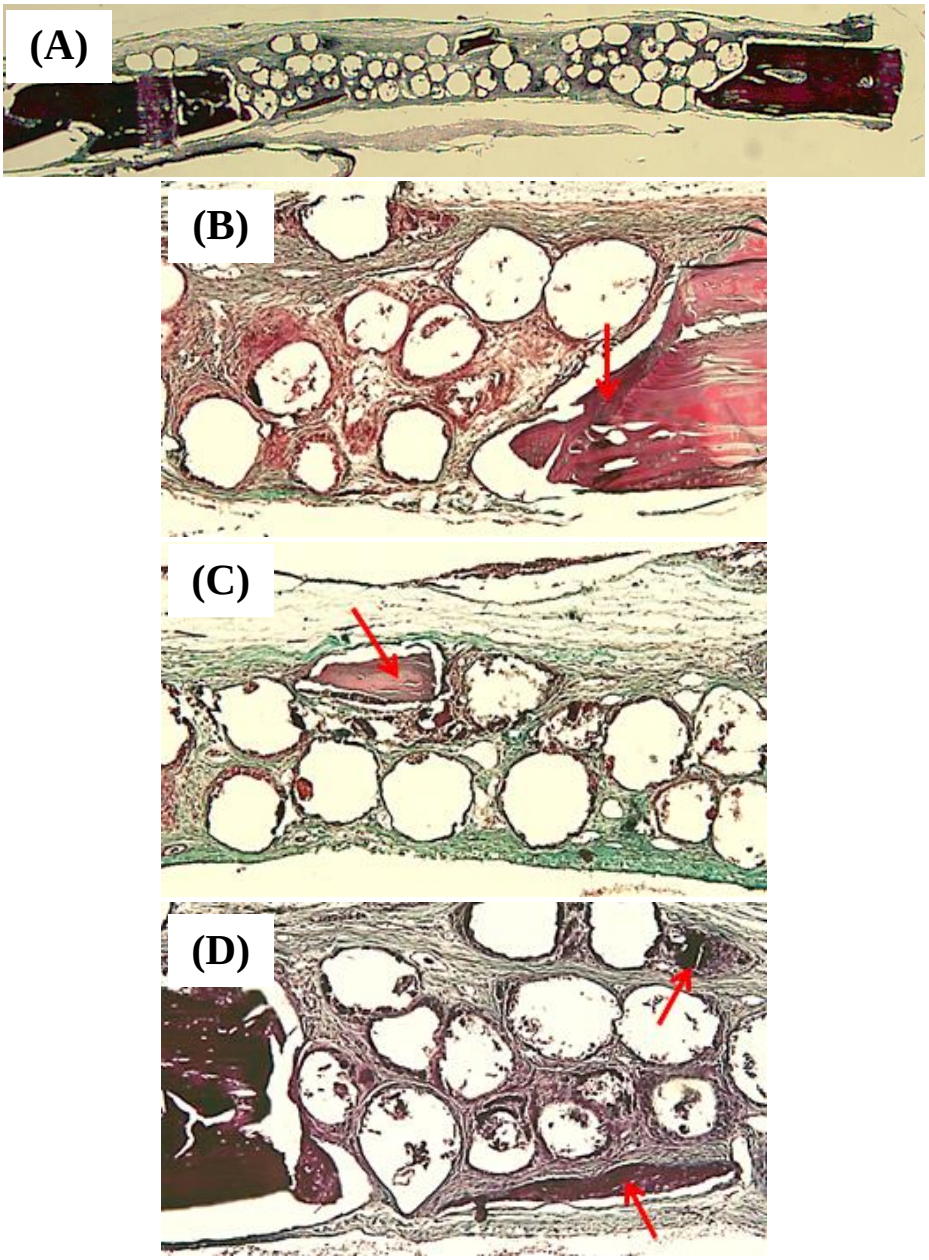

Figure 21.(A) lower magnification and (B)-(D) higher magnification histomorphometric images of stained sections of microspheres with BMP. (Arrows indicate new bone).

Chapter 4.

Conclusion

4.1. Enhancing Biocompatibility of Bone Cement Microspheres through Rapid Apatite Coating

Calcium phosphate microspheres were successfully fabricated by water/oil emulsion of bone cement paste. In addition of TTCP, their mechanical property was increased by around 30%. Also, fabrication time was extremely reduced from 1 week to 2hours through rapid apatite coating. Since previous studies tried to fully convert microspheres to HA in SBF solution, it took 1 week. However, this study intensively focused on modifying only the surface with concentrated SBF solution. More importantly, the coated microspheres exhibited significantly enhanced biological properties compared to conventionally made microspheres. This is because the stable HA coating layer stimulated the reaction inside of the spheres and resulted in superior microspheres with abundant HA. Therefore, the microspheres with apatite coating layer have great potential to be used as bone filler in dentistry and orthopedics.

4.2. Sustained Release of Biological Molecules from Hydroxyapatite Microspheres

Protein was effectively loaded in calcium phosphate microspheres by mixing bone cement powder and protein solution. After that, microspheres were kept in oil for 3 days in order to convert them into bioactive poor crystalline HA. Compared to conventional drug coating method, this method provides much more preferable release behavior since the proteins in the microspheres found to be continuously released more than at least one month. In preliminary *in vivo* animal test, new bones were well grown into the defect area where BMP loaded microspheres were. In addition to osetoconductivity of HA itself, BMP led to osetoinductivity, resulting in accelerated new bone formation. Therefore, microspheres fabricated from this experiment have great potential as a promising drug release carrier for bone healing applications.

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초록

골 대체재료의 수요가 늘어남에 따라 생체활성 세라믹이 치과와 정형외과에서 널리 사용되고 있다. 특히, 하이드록시아파타이트는 생체적합성이 뛰어나 유망한 골 결함용 임플란트재료로서 점점 주목을 받고 있다. 하지만 많은 우수한 특성에도 불구하고 밀한 하이트록시아파타이트는 임플란트재료로서 제한이 있다. 이는 생분해성이 매우 낮고 새로운 뼈가 차들어 갈만한 충분한 공간이 없기 때문이다. 이러한 밀한 하이드록시아파타이트의 문제점을 해결하기 위해 미립구형태의 하이트록시아파타이트가 개발되었다. 미립구는 외과적인 수술을 최소화하는 주사성을 가능하게 하고 또한 미립구 사이의 공간으로 골 조직이 차들어 갈 수 있게 해준다. 골의 회복속도를 증가시키기 위해 약물이나 생체활성 단백질을 세라믹에 담지하지만, 세라믹제조 공정의 열처리는 이를 제한시킨다.

이러한 문제를 극복하기 위한 방법으로 상온에서 제조가 가능한 하이드록시아파타이트 미립구를 만들기위해 본시멘트가 도입이 되었다. 연구자들이 이미 본시멘트를 이용해 미립구를 만들었지만 그것들은 제조공정시간과 약물담지에 있어서 낮은 효율성을 가지고 있다. 알파-인산삼칼슘을 이용한 기존의 연구에서는 기계적 안

정성과 화학적 안정성을 얻기위해미립구를 생체모사용액에 일주일 정도 담가 놓아야 했다. 이는 두가지의 문제점이 있다. 첫째로는, 연구자에게 있어 시간이 너무 오래걸린다는 것이다. 두번째는, 약물이 미립구 내에 담지가 되면 일주일간 생체모사용액에서 많은 양의 약물이 손실된다는 것이다.

첫번째 연구에서, 생체모사용액 없이 기계적 안정성을 얻기 위해 본시멘트 재료로 인산사칼슘과 경화액으로서 구연산이 추가로 사용되었다. 화학적 안정성을 수 시간 내로 얻기 위해 10배 농도가 높은 생체모사용액과 비슷한 용액으로 미립구의 표면을 아파타이트로 코팅을 하였다. 그래서 최종적으로 기계적, 화학적으로 안정성을 갖고 표면처리가 된 인산칼슘 미립구를 만들었다. 인산사칼슘이 강도의 차이를 만드는데 중요한 역할을 했다. 인산사칼슘이 알파-인산삼칼슘보다 열배정도 잘 녹기 때문에 빨리 녹아 아직 녹지않은 알파-인산삼칼슘 입자 사이를 채웠다. 또한, 분화전의 골세포를 이용한 세포실험에서 아파타이트코팅층이 있는 것이 코팅층이 없는 것에 비해 증진된 생체적합성을 보여주었다. 더 나은 특성을 가진 미립구를 훨씬 빠른시간안에 제조했다는 것에서 의미가 있었던 실험이었다.

첫 번째 연구에서 제조시간을 눈에 띄게 줄였지만, 약물이 손실되는 몇 시간의 담금시간이 있기 때문에 약물전달 응용분야에는 적절하지 않을 것이다. 그래서 약물이 담지된 양을 예측하기가 어렵고 정확한 약물 복용량을 필요로 하는 생체의료산업에 부적절하다. 친수성용액에서 담금을 피하면서 화학적 안정성을 동시에 얻기 위해 미립구가 기름 내부에 보관이 되어 경화액의 수분으로 하이드록시아파타이트로 변환이 되었다. 3일 후에, 반응물들이 많이 소모가 되었고 하이드록시아파타이트가 주요한 상이 되었다. 시각화할 수 있는 장점 때문에 약물이나 성장인자 대신 녹색형광단백질이 사용되었다. 녹색형광단백질이 미립구 내부에 잘 분산이 되어있었고 방출거동이 적어도 한 달은 지속되는 것으로 보였다. 생체의료분야에서의 잠재적 사용가능성을 확인하기 위해 쥐를 사용해 예비적인 동물실험을 하였다. 뼈형성단백질이 미립구내부에 담지되었고 담지되지 않은 미립구보다 더 좋은 골유도성과 골전도성을 보였다.

결론적으로, 이러한 두 실험은 시간적으로 그리고 약물담지에서 높은 효율성을 가진 안정한 인산칼슘계 미립구를 만드는 것에 초점을 두었다. 세포실험은 급속히 형성된 아파타이트 코팅층이 효과적으로 생체적합성을 향상시킨 것을 보였다. 또한, 예비 동물실험은 기름에서 변환된 인산칼슘계 미립구가 약물을 담지하고 골충전

재로 쓰일 수 있는 가능성을 입증했다.

주요어: 미립구, 하이드록시아파타이트, 인산칼슘계 본시멘트, 물/기름 에멀젼, 지속적 약물 방출, 골재생, 생체실험.

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